

ISOLATION, CULTIVATION, AND *IN VITRO* SUSCEPTIBILITY TESTING OF *BORRELIA BURGDORFERI* SENSU LATO: A REVIEW

GORANA VEINOVIĆ, BRANKICA FILIPIĆ and JELENA STANKOVIĆ

Department of Microbiology and Immunology, Faculty of Pharmacy, University of Belgrade, 11000 Belgrade, Serbia

Abstract – Lyme borreliosis is the most common vector-borne disease in the northern hemisphere. The agents of Lyme borreliosis are borrelia, bacteria of the family *Spirochaetaceae*, which are grouped in *Borrelia burgdorferi* sensu lato species complex. Borreliae are fastidious, slow-growing and biochemically inactive bacteria that need special attention and optimal conditions for cultivation. The isolation of *Borrelia* from clinical material and their cultivation is a time-consuming and demanding procedure. Cultivation lasts from 9 up to 12 weeks, which is much longer than is necessary to grow most other human bacterial pathogens. Although *B. burgdorferi* sensu lato is susceptible to a wide range of antimicrobial agents *in vitro*, up to now the susceptibility of individual *Borrelia* species to antibiotics is defined only partially.

Key words: *Borrelia*, growth conditions *in vitro*, susceptibility testing

INTRODUCTION

Lyme borreliosis is a multisystem disease caused by spirochete *B. burgdorferi* sensu lato that is transmitted to humans by ticks of the *Ixodes* complex and is manifested with diverse clinical signs and symptoms and with several variations in the course of the disease. A complete presentation of the disease has been divided into three clinical stages: early infection manifested as skin lesion erythema migrans at the site of the tick bite (stage 1), followed by involvement of the nervous system, joints and/or heart (stage 2) and late involvement of the nervous system, joints and skin that appear within months or years (Stanek and Strle, 2003; Steere, 2001).

At least four *B. burgdorferi* sensu lato species, *B. burgdorferi* sensu stricto, *B. afzelii*, *B. garinii*, and *B. spielmanii* are pathogenic to humans in Europe; *B. bissettii* and *B. lusitaniae* are rarely, while *B. valaisi-*

ana is considered as potential cause of human disease in Europe. The only known species that causes human infection in North America is *B. burgdorferi* sensu stricto, while *B. garinii* and *B. afzelii* have been found in Asia (Bergström et al., 2002; Colares-Pereira et al., 2004; Fingerle et al., 2008; Rijpkema et al., 1997; Stanek and Strle, 2003; Steere, 2001). Skin manifestations (erythema migrans and acrodermatitis chronica atrophicans) and nerve involvement (Lyme neuroborreliosis) are frequently associated with *B. afzelii* and *B. garinii* respectively, while joint involvement (Lyme arthritis) is more often observed in the USA than in Europe and is associated with *B. burgdorferi* sensu stricto (Stanek and Strle, 2003; Steere, 2001; Strle et al., 1996a). In Europe the most frequently isolated species from human material is *B. afzelii*, followed by *B. garinii*, while *B. burgdorferi* sensu stricto is a rarely isolated species (Ružić-Sabljić et al., 2002; Stanek and Strle, 2003; Strle et al., 1999a; Strle et al., 2011).

Geographic distribution and incidence rate

Lyme borreliosis occurs in North America (from the Mexican border in the south to the southern Canadian provinces in the north), the whole Europe, parts of North Africa (Maghreb), and northern Asia (Russian Siberia and the Far East, Sakhalin, Japan, China and Korea). In North America, Lyme borreliosis has been recorded in almost all states. The existence of Lyme borreliosis in the southern hemisphere (South and Central America, Sub-Saharan Africa, southern Asia, Australia) has never been reliably confirmed (Hubálek, 2009).

The incidence of Lyme borreliosis is associated with the prevalence of the main vectors – ticks, which are classified as the *Ixodes ricinus* complex, also called *Ixodes persulcatus* complex (Hubálek, 2009; Steere et al., 2004). Species of borrelia (pathogenic, less pathogenic and non-pathogenic *Borrelia* species) and geographical distribution of ticks are presented in Table 1.

About 85,000 and 19,000 (from 15,000 to 20,000) cases are reported annually in Europe and in the USA, respectively (estimated from available national data) (Lindgren and Jaenson, 2006; Steere, 2001). The highest incidence of Lyme borreliosis is in central Europe (e.g. Slovenia, 155/100,000) and the lowest in the UK (0.7/100,000) and Ireland (0.6/100,000) (Lindgren and Jaenson, 2006).

The registration of patients with Lyme borreliosis is necessary in only a few European countries, and it is assumed that the real incidence of this disease is most probably higher.

Causative agent

Borreliae are thin, elongated, and motile bacteria with 7 to 11 periplasmic flagella, which are enclosed between the outer and cytoplasmic membranes. The cells are 8 to 22 μm long, 0.25-0.30 μm wide and composed of 3-10 loose coils (Barbour and Hayes, 1986; Preac-Mursic and Wilske, 1993). The helical shape of *Borrelia burgdorferi* sensu lato (vis-

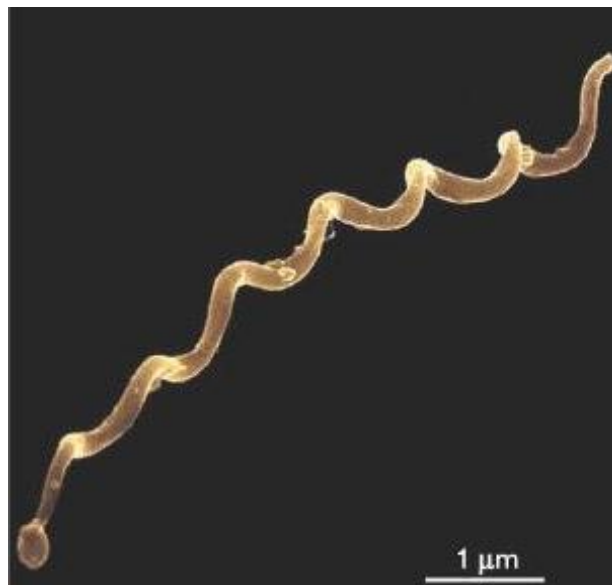


Fig. 1. Scanning electron micrograph of *Borrelia burgdorferi* sensu lato (Rosa et al., 2005)

ible in the scanning electron micrograph) is shown in Fig. 1 (Rosa et al., 2005).

The outer cell membrane is very fluid and contains transmembrane and outer surface proteins (Osp). Outer surface proteins (Osp A, Osp B, Osp C, Osp D, Osp E in Osp F) are lipoproteins encoded by the linear and circular plasmids. They are important for maintaining the structure of the membrane, while some of them have a role in enzymatic reactions or in transport through the membrane. Osp A, Osp B, and Osp C are the most important antigens of *Borrelia*. On their surface are many antigenic characteristics, which give to *Borrelia* the properties of heterogeneous bacteria (Dressler et al., 1993; Wilske et al., 1988). In addition, the outer membrane also contains lipopolysaccharides that are similar to the lipopolysaccharides of Gram-negative bacteria (Barbour and Hayes, 1986; Cox et al., 1996). Different outer surface proteins help *Borrelia* in adaptation and survival in different arthropod and mammalian environments (de Silva and Fikrig, 1997). Osp A is one of the major outer membrane lipoproteins of *Borrelia* and has been used for serological diagnosis as well as for vaccine development (Hilton et al., 1996; Kramer et al. 1996).

Table 1. Species of borrelia and geographical distribution of ticks (Steere et al., 2004; Stanek and Strle 2003).

<i>Borrelia</i> species	Tick (the main vector of Lyme borreliosis)	Location
Pathogenic		
<i>B. afzelii</i>	<i>I. ricinus</i>	Europe
	<i>I. persulcatus</i>	Asia
<i>B. garinii</i>	<i>I. ricinus</i>	Europe
	<i>I. persulcatus</i>	Asia
<i>B. burgdorferi</i> sensu stricto	<i>I. scapularis</i>	Eastern and central parts of North America
	<i>I. pacificus</i>	Western part of North America
	<i>I. ricinus</i>	Europe
<i>B. spielmanii</i>	<i>I. ricinus</i>	Europe
Less pathogenic and non-pathogenic		
<i>B. andersonii</i>	<i>I. dentatus</i>	Eastern part of North America
<i>B. bissettii</i>	<i>I. spinipalpis</i>	Western part of North America
	<i>I. pacificus</i>	
<i>B. valaisiana</i>	<i>I. ricinus</i>	Central Europe, Ireland, Great Britain
<i>B. lusitaniae</i>	<i>I. ricinus</i>	Europe (mainly Portugal)
<i>B. japonica</i>	<i>I. ovatus</i>	Japan
<i>B. tanukii</i>	<i>I. tanukii</i>	Japan
<i>B. turdae</i>	<i>I. turdus</i>	Japan
<i>B. sinica</i>	<i>I. persulcatus</i>	China

Table 2. The main differences between MKP, BSK-II and BSK-H media.

Medium	Gelatin	Yeast extract	Glucose (%)	Rabbit serum
BSK-II	present	present	5	6 % non-inactivated
MKP	present	non-present	3	7,2 % heat-inactivated
BSK-H	non-present	present	6	6 % non-inactivated

Antibodies directed against Osp A appear in the later stage of Lyme borreliosis (Wilske et al., 1996). Osp B has adhesive properties like Osp A, and antibodies directed against this antigen appear in the late stage of disease (Dressler et al., 1993). Osp C is the predominant seroreactive antigen in the early stage of Lyme borreliosis, and important in the transmission of the spirochete from tick to mammal. Rapid synthesis of Osp C by *Borrelia* during tick feeding is an essential

in the capacity of *Borrelia* to infect mammalian hosts, including humans, when transmitted by ticks, and after transmission from the tick may play a role in colonization of host tissues (Schwan et al., 1995; Schwan, 2003). The sequences of Osp C vary among strains and only a few of the groups of sequences are associated with dissemination, and Osp C alleles have been linked to infectivity as well as to invasiveness (Lagal et al., 2003; Seinost et al. 1999).

The genome of *Borrelia* species consists of a linear chromosome of approximately one million base pairs (Mb) and various linear and circular plasmids (Saint Girons et al., 1992). *B. burgdorferi* sensu stricto strain B31 was the first spirochete whose complete genome was sequenced. The genome of this strain consists of a linear chromosome of 910,725 bp and 17 linear and circular plasmids of 533,000 bp (Fraser et al., 1997).

The chromosome of *Borrelia* is important for the identification of species, and methods for identification are based on the analysis of plasmid or chromosomal DNA (e.g. restriction of entire DNA with different restriction enzymes, hybridization, DNA sequencing, etc.) (Belfaiza et al., 1993; Picken et al., 1996; Wang et al., 1999). *B. burgdorferi* sensu lato strains differ among themselves in relation to different plasmid profiles. They have an unusual plasmid content of linear and circular plasmids that may vary in number and size. This feature is very important in comparing and distinguishing strains within the same species (Xu and Johnson, 1995).

The spreading of *Borrelia* through the skin and other tissue is facilitated by the binding of human plasminogen, which can then be converted to active plasmin. *Borrelia* with bound plasmin is able to degrade fibronectin, penetrate the endothelium, and activate matrix metalloprotease-9 (MMP-9), and collagenase 1 (MMP-1) (Coleman et al., 1995; Coleman et al., 1999; Gebbia et al., 2001).

Isolation of B. burgdorferi sensu lato from clinical materials and growth conditions in vitro

The isolation of *Borrelia* from clinical materials is a golden standard for confirming borrelial infection, especially useful during the first several weeks of infection when serodiagnostic tests are insensitive (Ružić-Sabljić et al., 2006; Steere, 2001).

Borrelia can be isolated from different clinical materials such as skin, blood, cerebrospinal fluid (CSF), etc. during early as well as chronic stages of Lyme borreliosis (Ružić-Sabljić et al., 2002; Wilske

and Preac-Mursic, 1993). The clinical material for isolation must be taken from patients under aseptic conditions, before antimicrobial therapy, in as large a quantity as possible (e.g. 2 mL of CSF, 10mL of blood) and inoculated into the medium as soon as possible (Wilske and Preac-Mursic, 1993; Wilske and Pfister, 1995). In addition to the above-mentioned, temperature is very important during the transportation of clinical material from patient to laboratory. Room temperature was described as suitable for the transport of samples infected with *Borrelia* during the period from one to 11 days, while refrigerator temperature (5°C) was described as inadequate (Berger et al., 1992; Campbell et al., 1994).

Isolation, as well cultivation of borrelia, is a demanding, time-consuming and expensive procedure characterized by a modest level of sensitivity (Kollars et al., 1997; Maraspin et al., 2001; Nadelman et al., 1996; Strle et al., 1996a; Wormser et al., 2000a), and few laboratories are equipped to carry it out.

The yield of *Borrelia* culture from clinical samples is usually low, from 20 to 90% (usually 50%) of skin biopsy from erythema migrans (Nowakowski et al., 2001; Strle et al., 1996a; Wilske and Preac-Mursic, 1993), while the culture from blood and cerebrospinal fluid (CSF) yields borrelia in less than 10% of samples (Maraspin et al., 2002; Wilske and Preac-Mursic, 1993).

Borreliae are fastidious, slow-growing, and biochemically inactive bacteria that need special attention and optimal conditions for cultivation, and they grow best under anaerobic conditions at 30-34°C (Barbour and Hayes, 1986; Preac-Mursic et al., 1986; Preac-Mursic and Wilske, 1993). Some strains of *Borrelia* grow well at 35-39°C, while temperatures of 40°C and higher reduce or prevent their growth (Barbour, 1984; Heroldova et al., 1998; Hubalek et al., 1998; Reisinger et al., 1996). Generally, low temperatures (4°C) are better tolerated than high temperatures (37-42°C) (Preac-Mursic and Wilske, 1993).

The generation time of *Borrelia* is long and ranges from 7 to 20 h; it is influenced by available nutri-

ents, conditions of cultivation and the adaptation of *Borrelia* to the artificial medium (Preac-Mursic and Wilske, 1993). Cultivation lasts from 9 to 12 weeks, which is much longer than needed to grow most other human bacterial pathogens (Ružić-Sabjić et al., 2002; Wormser et al., 2000a). On the other hand, borrelia requires complex media for *in vitro* cultivation, due to their inability to synthesize any amino acids, nucleosides, nucleotides, fatty acids, or other cellular building blocks (Fraser et al., 1997).

Many factors can influence *in vitro* *Borrelia* growth, such as medium ingredients, pH of medium, temperature of incubation, contaminants, sample cell density, the capacity of particular borrelial species to grow, number of different *Borrelia* strains in the sample, previous antibiotic therapy, local anesthesia at the site of skin biopsy, size of skin biopsy specimens, and conditions during the transport of samples to the laboratory (Barbour 1984; Callister et al., 1990; Campbell et al., 1994; Hubálek et al., 1998; Jobe et al., 1993; Kollars et al., 1997; Pollack et al., 1993; Ružić-Sabjić et al., 2006; Wormser et al., 2000a; Yang et al., 2001).

Borrelia has an ability for *in vitro* transformation of normal, mobile spirochetes to cystic forms under unfavorable conditions in their environment (Brorson and Brorson, 1997). The authors evaluated the behavior of *Borrelia* under controlled conditions and *Borrelia* was cultivated in commercial BSK-H medium, which contained 6% rabbit serum, and in BSK-H medium without rabbit serum. In the medium without rabbit serum, borreliae were transformed into cystic forms, but after the cystic forms were transferred to the same culture medium with rabbit serum, they were transformed into regular, mobile spirochetes after 6 weeks, and their regeneration time was normal. This means that similar phenomenon may occur *in vitro* under other conditions unfavorable for *Borrelia* (e.g. the presence of antibiotics). Similarly, when normal, mobile spirochetes were inoculated into cerebrospinal fluid, the spirochetes were converted to cysts (spheroplast L-forms), but when these cystic forms were transferred to BSK-H medium, the cysts were converted back to

normal, mobile spirochetes after incubation. When neuroborreliosis is suspected, it is necessary to realize that *Borrelia* species can be present in cystic form, and these cysts have to be recognized by microscopy (Brorson and Brorson, 1998).

Description of culture media

The preparation of culture media is demanding and expensive. Different culture media have been introduced and evaluated for borrelial cultivation, but for routine work most frequently three liquid media were reported: modified Kelly-Pettenkofer (MKP), Barbour-Stoenner-Kelly II (BSK-II) medium and commercially available BSK-H (Sigma, USA) medium (Barbour 1984; Pollack et al., 1993; Preac-Mursic et al., 1986). The majority of the ingredients in these media are equivalent (e.g. CMRL as a source of amino acids, vitamins, and other factors, N-acetyl-D-glucosamine-a precursor for bacterial cell wall biosynthesis, HEPES, neopeptone, pyruvic acid, citric acid, bovine serum albumin, rabbit serum, etc.).

On the other hand, media differ with regard to concentration, origin (diverse commercial sources), and the preparation of certain ingredients (Barbour, 1984; Pollack et al., 1993; Preac-Mursic et al., 1986; Ružić-Sabjić et al., 2006). For example, in contrast to BSK-H, BSK-II and MKP contain gelatin, but MKP lacks yeast extract and contains a higher concentration of rabbit serum that differs in its preparation (7.2% heat-inactivated rabbit serum in MKP versus 6% non-inactivated in BSK-II and BSK-H). There is also a difference in glucose concentration between the three media: 3, 5 and 6% for MKP, BSK-II, and BSK-H medium, respectively. The main differences between MKP, BSK-II and BSK-H are presented in Table 2.

The source and quality of albumin and specific preparation of rabbit serum can influence *Borrelia* growth *in vitro* and some preparations of rabbit serum contain antispirochetal immunoglobulin G that reduces or inhibits the growth of *Borrelia* (Pollack et al., 1993). Similarly, if identical BSK media contain different lots of bovine serum albumin from differ-

ent manufacturers, they differ in their ability to support the growth of a small number of *Borrelia* strains (Callister et al., 1990).

Borrelia strains can also grow on solid media with agarose to solidify the liquid media under microaerophilic or anaerobic conditions (De Martino et al., 2006; Kurtti et al., 1987; Preac-Mursic et al., 1991). By using a solid medium, distinct morphological variations in colonies of *B. burgdorferi* sensu stricto strains can be observed.

Three pathogenic *Borrelia* species (*B. afzelii*, *B. garinii*, and *B. burgdorferi* sensu stricto) grow well in both MKP and BSK-II media as was described previously (Ružić-Sabljić and Strle, 2004). On the other hand, Ružić-Sabljić et al. (2006) indicated a similar suitability of MKP and BSK-II media for routine laboratory work. The authors evaluated the isolation rate of *B. afzelii*, *B. garinii*, and *B. burgdorferi* sensu stricto from MKP and BSK-II media and showed comparable *Borrelia* isolation rates in both media.

Comparison of *B. afzelii*, *B. garinii*, and *B. burgdorferi* sensu stricto

B. burgdorferi sensu stricto has been described as a more virulent and aggressive *Borrelia* species than *B. afzelii* i *B. garinii* (Ružić-Sabljić and Strle 2004; Strle et al., 1999a, Strle et al., 2011).

Ružić-Sabljić and Strle (2004) compared the growth of *B. afzelii*, *B. garinii*, and *B. burgdorferi* sensu stricto in MKP and BSK-II media, and established that in a mixture of two species, *B. burgdorferi* sensu stricto behaves as the most aggressive species, followed by *B. garinii* and at lastly *B. afzelii*. *B. burgdorferi* sensu stricto overgrew *B. afzelii* and *B. garinii* in both media, but the results were found to be statistically significant only for the MKP medium, while *B. garinii* overgrew *B. afzelii* but significant differences were established only for the BSK-II medium.

Strle et al. (1999a) compared patients in Europe and USA with culture-confirmed erythema migrans, and showed *B. burgdorferi* sensu stricto (iso-

lated from USA patients) to be more virulent than *B. afzelii* (isolated from Slovenian patients). Erythema migrans spreads more slowly, the duration is longer, and the possibility of dissemination is less common in European patients, unlike erythema migrans in USA patients, which spreads more rapidly, the duration is shorter, and it is associated with more intensive inflammation and signs that often indicate the dissemination of *Borrelia*.

Similarly, in a comparison of the European and USA patients with culture-confirmed erythema migrans, Strle et al. (2011) showed *B. burgdorferi* sensu stricto isolated from USA patients to be more virulent than *B. garinii* isolated from Slovenian patients. Slovenian patients with erythema migrans caused by *B. garinii* developed larger lesions than USA patients, but systemic symptoms and abnormal physical findings, such as fever or regional lymphadenopathy, appear more frequently in USA patients with erythema migrans caused by *B. burgdorferi* sensu stricto than in European patients.

Furthermore, *B. burgdorferi* sensu stricto induces normal macrophages to secrete higher levels of chemokines and cytokines than *B. afzelii* or *B. garinii*, indicating that *B. burgdorferi* sensu stricto induces a greater inflammatory response in macrophages than the other two *Borrelia* species (Strle et al., 2009).

In vitro susceptibility of *B. burgdorferi* sensu lato to different antimicrobial agents

Many *in vitro* studies have been performed to find an optimal treatment for Lyme borreliosis. Previous *in vitro* antimicrobial susceptibility studies have demonstrated the susceptibility of *B. burgdorferi* sensu lato to a broad spectrum of antimicrobial agents, including penicillin G, amoxicillin, azithromycin, erythromycin, clarithromycin, ceftriaxone, cefuroxime, cefotaxime, doxycycline, vancomycin, gemifloxacin etc. (Baradaran-Dilmaghani and Stanek, 1996; Dever et al., 1993; Hunfeld et al., 2000a; Hunfeld et al., 2000b; Kraiczy et al., 2001; Levin et al., 1993; Ružić-Sabljić et al., 2005; Sicklinger et al., 2003).

Published *in vitro* susceptibility results on the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of antimicrobial agents for borreliae are difficult to compare because of methodological differences. Up to now, broth microdilution and macrodilution methods in different media (MKP, Barbour-Stoener-Kelly, BSK-II or standardized BSK-H) with various inoculum concentrations (10^4 - 10^7 / mL), incubation periods (3-7 days for MIC, 1-6 weeks for MBC) were applied. Samples were mainly checked for the presence of *Borrelia* by the enumeration of cells and observation of borrelia motility using dark-field microscopy or by visual conformation of medium color changes in microtiter plates.

On the other hand, enumeration and observation of *Borrelia* is time-consuming and demanding, especially if more samples need to be checked simultaneously and subjectively.

Boerner et al. (1995) reported that the MIC values for *Borrelia* are significantly influenced by the density of the inoculum and the mode of MIC determination (microscopical and macroscopical MIC reading). The authors showed that differences between MIC values obtained by testing 10^6 versus 10^7 *Borrelia* cells/mL were on average equivalent to 1.2 dilution steps for the macroscopical but only 0.2 dilution steps for the microscopical method, and that the high agreement of microscopical MICs has been due to the standardized enumeration method. Using an inoculum of 10^6 cells/mL, MICs determined macroscopically (which is less time-consuming than the microscopical method) were significantly lower than MICs determined microscopically because the inoculum of 10^6 cells/mL was too small to induce a distinct color change and sediment formation, whereas borrelial growth was often detectable microscopically. On the other hand, when using 10^7 cells/mL as the final inoculum, 75.9% of the MICs revealed identical values for both the macroscopical and the microscopical reading modes.

This discrepancy in the methodology led to a wide range of published MIC and MBC results. For

example, for amoxicillin, ceftriaxone, doxycycline, and azithromycin the MIC varies from ≤ 0.03 to 4 mg/L, from ≤ 0.01 to 4 mg/L, from 0.06 to 4 mg/L, and from 0.003 to 0.06 mg/L, respectively. Similarly, for amoxicillin, ceftriaxone, doxycycline, and azithromycin, the MBC varies from ≤ 0.03 to >16 mg/L, from 0.006 to 4 mg/L, from 0.4 to 32 mg/L, and from 0.003 to 4 mg/L, respectively. (Baradaran-Dilmaghani and Stanek, 1996; Dever et al., 1992; Hunfeld et al., 2000a; Hunfeld et al., 2000b; Levin et al., 1993; Morgenstern et al., 2009; Ružić-Sabljić et al., 2005; Sicklinger et al., 2003). Despite the problems in the methodology, *in vitro* susceptibility testing has also been limited because of the small number of tested *B. burgdorferi* sensu lato strains.

Ružić-Sabljić et al. (2005) tested the susceptibility of *B. afzelii* strains to antimicrobial agents, and found that several isolates survived high concentrations of doxycycline, cefuroxime axetyl and amoxicillin and grew after 6 weeks but not after 3 weeks incubation. Their results showed that borrelia could survive exposure to antibiotics *in vitro*, but mechanisms of resistance are not obvious.

One possible explanation for the survival of *Borrelia* in the presence of antibiotics could be the ability of *Borrelia* to persist in a latent phase. The ability could enable spirochetes to survive the presence of antimicrobial agents in their environment and begin to re-grow under suitable conditions, e.g. after antibiotic elimination in the body or after reduction or loss of activity of antibiotics during a prolonged incubation period *in vitro*. On the other hand, Preac-Mursic et al. (1996b) showed that *Borrelia* has the ability to transform into a cystic, spherical form (spheroplasts, L-form), which may offer protection from antimicrobial agents, allowing them to survive unfavorable conditions such as the presence of antibiotics. The spheroplast-L-form without cell walls can be a possible reason why *Borrelia* can survive in an organism for a long time and the cell-wall-dependent antibody titers disappear and emerge after reversion.

Georgilis et al. (1992) reported that human skin fibroblasts could protect *Borrelia* from ceftriaxone *in*

vitro. While this antibiotic lost efficiency when *Borrelia* was cultured in the presence of cells, ceftriaxone was lethal for spirochetes in the absence of human skin fibroblasts. Similarly, the study of Brouqui et al. (1996) confirmed the ability of *Borrelia* to survive in human cells *in vitro* in the presence of antimicrobial agents in their environment. When *Borrelia* was cultivated in an axenic medium in the presence of penicillin G or ceftriaxone, the number of bacteria decreased rapidly, whereas then they were co-cultivated with eukaryotic cells in the presence of penicillin G or ceftriaxone, no change in the viable borrelial count was observed. Doxycycline and erythromycin were found to act efficiently against *Borrelia*, especially when they were grown in the presence of eukaryotic cells. No viable *Borrelia* was found after incubation with these antibiotics, indicating no protective effect of eukaryotic cells on doxycycline and erythromycin actions.

In some studies, antimicrobial agents (e.g. β -lactams) showed moderate or weak *in vitro* activity against *Borrelia*, but are mainly effective *in vivo*. Nevertheless, the *in vitro* activity of many antimicrobial agents against *Borrelia* has not always correlated with clinical experience (Hassler et al., 1990; Luft et al., 1989).

A possible interaction between the ingredients of media, such as bovine albumin and antimicrobial agents, as well as the poor chemical stability of some antibiotics during the prolonged incubation period necessary for *Borrelia* susceptibility testing, may lead to the reduction or loss of their activity (Boerner et al., 1995; Dever et al., 1992; Reisinger et al., 1995). On the other hand, inadequately prepared and stored antibiotic solutions may also be one of reasons for the weak *in vitro* activity of some antibiotics against *Borrelia* strains.

Before performing an antibiogram (*in vitro* susceptibility testing of antimicrobial agents against bacteria), it is necessary to prepare and store the antibiotic solution appropriately. Jorgensen and Turnidge (2003) indicated that tubes with antimicrobial agents should be tightly capped and stored

at 4 to 8°C until needed in order to minimize their evaporation and deterioration. The dilution of most antimicrobial agents should be used within 5 days of preparation and certain β -lactam antibiotics are too labile for prolonged storage in final concentration.

Dever et al. (1992) reported that penicillin concentration reduced during incubation period at 34°C in BSK-II medium. The levels of penicillin G were reduced to 17% and less than 2% of the initial concentration after 72 h and 7 days, respectively. Thus, concentration of penicillin was undetectable after 7 days of incubation, while the concentration of ceftriaxone was also diminished, but was still detectable (47% of the initial concentration remained) after the same period. Similarly, Kersten et al. (1995) reported decreased penicillin G concentrations of about 20% during 24 h of incubation; at 48 h more than 60% of the initial concentration was detectable at all the tested concentrations, while the remaining concentrations after 72 h were 50% and 20% of the initial concentration. After 48 h of incubation, doxycycline concentrations were more than 80% of initial concentrations. At 72 h, drug concentrations of between 64 and 76% of the initial values could be detected, indicating that doxycycline was quite stable under culture conditions.

The interaction between BSK-II medium and penicillin G led to a decrease in efficacy of 85.8% of this antibiotic after 72 h of incubation at 34°C, while other penicillins (mezlocillin and piperacillin) also showed a marked decrease with time of incubation. On the other hand, the activity of doxycycline and erythromycin increased against *Borrelia* strains when tested in BSK-medium (Boerner et al., 1995).

It is important to note that the effectiveness of some antibiotics such as penicillin and ceftriaxone *in vitro* and *in vivo* is temperature-dependent (Reisinger et al., 1996). The *in vitro* susceptibility of *Borrelia* strains to penicillin and ceftriaxone increased up to 16-fold after raising the temperature from 36°C to 38°C. This means that an increase in body tem-

perature may be beneficial during the antimicrobial treatment of Lyme borreliosis.

In order to provide constant and appropriate concentrations of antimicrobial agents for a prolonged incubation period, Stiernstedt et al. (1999) developed the dialysis culture method for the determination of MICs and MBCs of benzylpenicillin for *Borrelia*. In this method, borrelial suspensions were enclosed in sealed dialysis membrane bags and put into tubes with BSK medium with the appropriate two-fold serial dilution of the antibiotic, and control tubes with only BSK medium. The dialysis membrane bags were transferred every day for 6 days to new tubes with BSK medium and freshly added antibiotic, and the MIC was determined on day 7. However, a shortcoming of this method is that it is difficult to standardize.

Some studies reported differences in the antibiotic susceptibilities of pathogenic *Borrelia* species; MIC and MBC values may vary from one *Borrelia* species to another (Hunfeld et al., 2000a; Hunfeld et al., 2000b; Morgenstern et al., 2009; Preac-Mursic et al., 1996a; Sicklinger et al., 2003). In previous studies, *B. afzelii* and *B. burgdorferi* sensu stricto species were less susceptible to some antimicrobial agents, whereas *B. garinii* species was more susceptible than other *Borrelia* species to many antibiotics.

In the study of Hunfeld et al. (2000a), the MICs of penicillin for *B. afzelii* isolates were ten times higher than those for *B. burgdorferi* sensu stricto, *B. valaisiana*, and *B. bissettii* isolates, and 100 times higher than for other *Borrelia* isolates. Similarly, Hunfeld et al. (2000b) found that the MICs of amoxicillin were lower for *B. garinii* isolates than for *B. afzelii*, *B. burgdorferi* sensu stricto, and *B. valaisiana*, while the MICs of cefixime for *B. garinii* isolates proved to be lower than those for *B. afzelii* isolates.

Sicklinger et al. (2003) also reported differences in antibiotic susceptibility between *Borrelia* species. *B. burgdorferi* sensu stricto showed higher susceptibility to amoxicillin than *B. afzelii* and *B. garinii* isolates; *B. afzelii* was more susceptible to ceftriaxone than the

other two *Borrelia* species, while *B. garinii* proved to be the most susceptible to azithromycin.

Morgenstern et al. (2009) reported that macrolides (erythromycin and clarithromycin) and doxycycline showed the highest *in vitro* activity against *B. spielmanii* in contrast to *B. afzelii*, *B. garinii* and *B. burgdorferi* sensu stricto species, while *B. spielmanii* was less susceptible to amoxicillin than the other genospecies.

Preac-Mursic et al. (1996a) investigated the killing effect in MKP medium and human serum during a 72 h exposure to amoxicillin, doxycycline, cefotaxime, ceftriaxone, azithromycin and penicillin G used in the treatment of Lyme borreliosis. *B. afzelii* and *B. garinii* strains had different reactions to the antibiotics; *B. garinii* strains appeared to be more susceptible to antibiotics. Furthermore, the authors found that differences in antibiotic susceptibility also exist within a single species.

In contrast to the above-mentioned studies that showed differences in antibiotic susceptibility among *Borrelia* species, the study by Baradaran-Dilmaghani and Stanek (1996) did not. Different antimicrobial agents (azithromycin, amoxicillin, ceftriaxone, cefotaxime, doxycycline, penicillin G sodium, roxithromycin, and trimethoprim-sulfamethoxazole) were tested against thirty *B. afzelii*, *B. garinii* and *B. burgdorferi* sensu stricto strains from various sources (blood, cerebrospinal fluid-CSF, heart, skin and tick). MIC and MBC were determined after 72 h and 96 h of incubation, respectively, and did not show significant differences between the tested strains. Interestingly, a difference was found only for doxycycline. The MICs for *B. afzelii* isolates were lower than for *B. garinii* isolates, unlike the study of Sicklinger et al. (2003) where only doxycycline did not show any differences in its effect on *B. afzelii*, *B. garinii*, and *B. burgdorferi* sensu stricto.

Some studies have reported that in relapsed patients with early Lyme borreliosis, *Borrelia* isolates cultured after the conclusion of roxithromycin (Hansen et al., 1992), ceftriaxone (Pfister et al., 1991)

and azithromycin (Hunfeld et al., 2005) treatment remained susceptible to these agents *in vitro*. On the other hand, Terekhova et al. (2002) reported susceptibility testing of laboratory strains and clinical isolates of *Borrelia* and demonstrated the existence of resistance to erythromycin in them. The results of this study indicated an important heterogeneity in the susceptibility of *B. burgdorferi* strains to erythromycin and suggested that erythromycin resistance could develop in *Borrelia* strains isolated from Lyme borreliosis patients that have been pre-exposed to the antibiotic, based on the existence of resistant subpopulations *in vitro*.

In order to overcome methodological problems, Hunfeld et al. (2000a) introduced a colorimetric microdilution method for *in vitro* susceptibility testing of *B. burgdorferi* *sensu lato* against antimicrobial agents. For this susceptibility testing, the final inoculum concentration was 10^6 cells/mL. This is the standardized test based upon color changes (occurring in the presence of phenol red) and result from the accumulation of nonvolatile acid produced by actively metabolizing spirochetes after 72 h of incubation. Growth of *Borrelia* was detected by software-assisted kinetic measurement of the decrease of absorbance. In this method, the growth of samples and controls was determined for each well, based on the decrease in absorbance ($A_{562/630}$) after 72 h (E_{72}) in comparison to the initial absorbance values (E_{10}). In mathematical terms, if the absorbance values at 72 h decreased 5% or more compared with the initial absorbance values, the well was considered positive for borrelial growth ($E_{72} < E_0$ minus 5%). The MIC was determined as the lowest concentration of antimicrobial agent with which no such significant color shift (decline of curve) could be detected. Colorimetric *in vitro* susceptibility testing has also been used in some other studies (Hunfeld et al., 2000b; Hunfeld et al. 2005; Kraiczky et al., 2001; Morgenstern et al., 2009). This MIC method is reliable, reproducible and convenient and can handle large numbers of isolates and antibiotics (Hunfeld et al., 2000a), but the problem related to its wider use is its unavailability to other laboratories.

Despite the fact that there are differences in the experimental conditions and test methods applied during *in vitro* susceptibility testing of antimicrobial agents against *Borrelia*, this bacterium highly susceptible to antibiotic treatment and the majority of patients with early Lyme borreliosis profit from the recommended antibiotic treatment (Girschick et al., 2009; Strle, 1999b; Wormser et al., 2000b). Treatment of early Lyme borreliosis, such as skin lesion erythema migrans, with β -lactam agents (such as amoxicillin and cefuroxime) and tetracycline agents was described as very successful in >90% of the cases (Smith et al., 2002; Thanassi and Schoen, 2000). About 5 to 10% of patients with erythema migrans fail to respond to antibiotic therapy. This is more common with macrolide agents than with β -lactam and tetracycline agents (Hansen et al., 1992; Hunfeld et al., 2002; Luft et al., 1996; Smith et al., 2002; Wormser et al., 2000b).

Some patients develop chronic persistent disease, and borreliae can be isolated in spite of previous treatment with antibiotics (Maraspin et al., 1995). Clinical and experimental data showed that after so-called adequate treatment in patients with Lyme borreliosis, *Borrelia* could persist in tissue, and failures of treatment have been reported for almost every suitable antimicrobial agent (Hassler et al., 1990; Preac-Mursic et al., 1989; Preac-Mursic et al., 1996a; Straubinger et al., 1997; Strle et al., 1993; Strle et al., 1996b; Wormser et al., 2003). In some cases, the failure of treatment could be due to irreversible tissue damage during active borrelial infection or inflammation in association with the infection, the induction of autoimmune mechanisms, and possible misdiagnosis (Strle, 1999b). In any case, treatment with antibiotics is reasonable at all stages of Lyme borreliosis infection and for all clinical manifestations; recommendations for the treatment have been reported previously (Girschick et al., 2009; Steere, 2001; Strle, 1999b).

Acknowledgments - The Ministry of Education and Science of the Republic of Serbia funded this work, grant No.: 175011.

REFERENCES

- Baradaran-Dilmaghani, R., and G. Stanek (1996). *In vitro* susceptibility of thirty *Borrelia* strains from various sources against eight antimicrobial chemotherapeutics. *Infection*. **24**, 60-63.
- Barbour, A.G. (1984). Isolation and cultivation of Lyme disease spirochetes. *Yale. J. Biol. Med.* **57**, 521-525.
- Barbour, A.G., and S.F. Hayes (1986). Biology of *Borrelia* species. *Microbiol. Rev.* **50**, 381-400.
- Belfaiza, J., Postic, D., Bellenger, E., Baranton, G., and I.S. Girons (1993). Genomic fingerprinting of *Borrelia burgdorferi* sensu lato by pulsed-field gel electrophoresis. *J. Clin. Microbiol.* **31**, 2873-2877.
- Berger, B.W., Johnson, R.C., Kodner, C., and L. Coleman (1992). Cultivation of *Borrelia burgdorferi* from erythema migrans lesions and perilesional skin. *J. Clin. Microbiol.* **30**, 359-361.
- Bergström, S., Noppa, L., Gylfe, Å., and Y. Östberg (2002). Molecular and cellular biology of *Borrelia burgdorferi* sensu lato, In: Lyme borreliosis: biology, epidemiology and control (Eds. J. Gray, O. Kahl, R.S. Lane, and G. Stanek), 47-90. CABI Publishing, New York.
- Boerner, J., Failing, K., and M.M. Wittenbrink (1995). *In vitro* antimicrobial susceptibility testing of *Borrelia burgdorferi*. Influence of test conditions on minimal inhibitory concentration (MIC) values. *Zbl. Bakt.* **283**, 49-60.
- Brorson, O., and S.H. Brorson (1997). Transformation of cystic forms of *Borrelia burgdorferi* to normal, mobile spirochetes. *Infection*. **25**, 240-246.
- Brorson, O., and S.H. Brorson (1998). *In vitro* conversion of *Borrelia burgdorferi* to cystic forms in spinal fluid, and transformation to mobile spirochetes by incubation in BSK-H medium. *Infection*. **26**, 144-150.
- Brouqui, P., Badiaga, S., and D. Raoult (1996). Eucaryotic cells protect *Borrelia burgdorferi* from the action of penicillin and ceftriaxone but not from the action of doxycycline and erythromycin. *Antimicrob. Agents. Chemother.* **40**, 1552-1554.
- Callister, S.M., Case, K.L., Agger, W.A., Schell, R.F., Johnson, R.C., and J.L. Ellingson (1990). Effects of bovine serum albumin on the ability of Barbour-Stoenner-Kelly medium to detect *Borrelia burgdorferi*. *J. Clin. Microbiol.* **28**, 363-365.
- Campbell, G.L., Piesman, J., Mitchell, P.D., Quan, T.J., Reed, K.D., and D.T. Dennis (1994). An evaluation of media for transport of tissues infected with *Borrelia burgdorferi*. *Am. J. Clin. Pathol.* **101**, 154-156.
- Coleman, J.L., Sellati, T.J., Testa, J.E., Kew, R.R., Furie, M.B., and J.L. Benach (1995). *Borrelia burgdorferi* binds plasminogen, resulting in enhanced penetration of endothelial monolayers. *Infect. Immun.* **63**, 2478-2484.
- Coleman, J.L., Roemer, E.J., and J.L. Benach (1999). Plasmin-coated *Borrelia burgdorferi* degrades soluble and insoluble components of the mammalian extracellular matrix. *Infect. Immun.* **67**, 3929-3936.
- Collares-Pereira, M., Couceiro, S., Franca, I., Kurtenbach, K., Schäfer, S.M., Vitorino, L., Gonçalves, L., Baptista, S., Vieira, M.L., and C. Cunha (2004). First isolation of *Borrelia lusitaniae* from a human patient. *J. Clin. Microbiol.* **42**, 1316-1318.
- Cox, D.L., Akins, D.R., Bourell, K.W., Lahdenne, P., Norgard, M.V., and J.D. Radolf (1996). Limited surface exposure of *Borrelia burgdorferi* outer surface lipoproteins. *Proc. Natl. Acad. Sci. U S A.* **93**, 7973-7978.
- De Martino, S.J., Sordet, C., Piémont, Y., Ružić-Sabljić, E., Thadée Vetter, M., Monteil, H., Sibilia, J., and B. Jaulhac (2006). Enhanced culture of *Borrelia garinii* and *Borrelia afzelii* strains on a solid BSK-based medium in anaerobic conditions. *Res. Microbiol.* **157**, 726-729.
- de Silva, A.M., and E. Fikrig (1997). Arthropod- and host-specific gene expression by *Borrelia burgdorferi*. *J. Clin. Invest.* **99**, 377-379.
- Dever, L.L., Jorgensen, J.H., and A.G. Barbour (1992). *In vitro* antimicrobial susceptibility testing of *Borrelia burgdorferi*: A microdilution MIC method and time-kill studies. *J. Clin. Microbiol.* **30**, 2692-2697.
- Dever, L.L., Jorgensen, J.H., and A.G. Barbour (1993). *In vitro* activity of vancomycin against the spirochete *Borrelia burgdorferi*. *Antimicrob. Agents. Chemother.* **37**, 1115-1121.
- Dressler, F., Whalen, J.A., Reinhardt, B.N., and A.C. Steere (1993). Western blotting in the serodiagnosis of Lyme disease. *J. Infect. Dis.* **167**, 392-400.
- Fingerle, V., Schulte-Spechtel, U.C., Ružić-Sabljić, E., Leonhard, S., Hofmann, H., Weber, K., Pfister, K., Strle, F., and B. Wilske (2008). Epidemiological aspects and molecular characterization of *Borrelia burgdorferi* s.l. from southern Germany with special respect to the new species *Borrelia spielmanii* sp. nov. *Int. J. Med. Microbiol.* **298**, 279-290.
- Fraser, C.M., Casjens, S., Huang, W.M., Sutton, G.G., Clayton, R., Lathigra, R., White, O., Ketchum, K.A., Dodson, R., Hickey, E.K., Gwinn, M., Dougherty, B., Tomb, J.F., Fleischmann, R.D., Richardson, D., Peterson, J., Kerlavage, A.R., Quackenbush, J., Salzberg, S., Hanson, M., van Vugt, R., Palmer, N., Adams, M.D., Gocayne, J., Weidman, J., Utterback, T., Watthey, L., McDonald, L., Artiach, P., Bowman, C., Garland, S., Fuji, C., Cot-

- ton, M.D. Horst, K. Roberts, K. Hatch, B. Smith, H.O., and J.C. Venter (1997). Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. *Nature*. **390**, 580-586.
- Gebbia, J.A., Coleman, J.L., and J.L. Benach (2001). *Borrelia* spirochetes upregulate release and activation of matrix metalloproteinase gelatinase B (MMP-9) and collagenase 1 (MMP-1) in human cells. *Infect. Immun.* **69**, 456-462.
- Georgilis, K., Peacocke, M., and M.S. Klempner (1992). Fibroblasts protect the Lyme disease spirochete, *Borrelia burgdorferi*, from ceftriaxone in vitro. *J. Infect. Dis.* **166**, 440-444.
- Girschick, H.J., Morbach, H., and D. Tappe (2009). Treatment of Lyme borreliosis. *Arthritis. Res. Ther.* **11**, 258.
- Hansen, K., Hovmark, A. Lebech, A.M. Lebech, K. Olsson, I. Halkier-Sørensen, L. Olsson, E., and E. Asbrink (1992). Roxithromycin in Lyme borreliosis: discrepant results of an *in vitro* and *in vivo* animal susceptibility study and a clinical trial in patients with erythema migrans. *Acta. Derm. Venereol.* **72**, 297-300.
- Hassler, D., Zöller, L. Haude, M. Hufnagel, H.D. Heinrich, F., and H.G. Sonntag (1990). Cefotaxime versus penicillin in the late stage of Lyme disease-prospective, randomized therapeutic study. *Infection.* **18**, 16-20.
- Heroldová, M., Němec, M., and Z. Hubálek (1998). Growth parameters of *Borrelia burgdorferi* sensu stricto at various temperatures. *Zentralbl. Bakteriol.* **288**, 451-455.
- Hilton, E., Devoti, J., and S. Sood (1996). Recommendation to include OspA and OspB in the new immunoblotting criteria for serodiagnosis of Lyme disease. *J. Clin. Microbiol.* **34**, 1353-1354.
- Hubálek, Z., Halouzka, J., and M. Heroldová (1998). Growth temperature ranges of *Borrelia burgdorferi* sensu lato strains. *J. Med. Microbiol.* **47**, 929-932.
- Hubálek, Z. (2009). Epidemiology of lyme borreliosis. *Curr. Probl. Dermatol.* **37**, 31-50.
- Hunfeld, K.P., Kraiczy, P. Wichelhaus, T.A. Schäfer, V., and V. Brade (2000a). New colorimetric microdilution method for *in vitro* susceptibility testing of *Borrelia burgdorferi* against antimicrobial substances. *Eur. J. Clin. Microbiol. Infect. Dis.* **19**, 27-32.
- Hunfeld, K.P., Kraiczy, P. Wichelhaus, T.A. Schäfer V., and V. Brade (2000b). Colorimetric *in vitro* susceptibility testing penicillins, cephalosporins, macrolides, streptogramins, tetracyclines, and aminoglycosides against *Borrelia burgdorferi* isolates. *Int. J. Antimicrob. Agents.* **15**, 11-17.
- Hunfeld, K.P., Kraiczy, P. Kekoukh, E. Schäfer, V., and V. Brade (2002). Standardised *in vitro* susceptibility testing of *Borrelia burgdorferi* against well-known and newly developed antimicrobial agents – possible implications for new therapeutic approaches to Lyme disease. *Int. J. Med. Microbiol.* **33**, 125-137.
- Hunfeld, K.P., Ružić-Sabljić, E. Norris, D.E. Kraiczy, P., and F. Strle (2005). *In vitro* susceptibility testing of *Borrelia burgdorferi* sensu lato isolates cultured from patients with erythema migrans before and after antimicrobial chemotherapy. *Antimicrob. Agents. Chemother.* **49**, 1294-1301.
- Jobe, D. A., Callister, S. M., and R. F. Schell (1993). Recovery of *Borrelia burgdorferi* by filtration. *J. Clin. Microbiol.* **31**, 1896-1898.
- Jorgensen, J.H., and J.D. Turnidge (2003). Susceptibility test methods: dilution and disk diffusion methods, In: Manual of clinical microbiology (Eds. P.R. Murray, E.J. Baron, M. A. Pfaller, F.C. Tenover, and R.H. Tenover), 1108-1127. American Society of Microbiology Press, Washington.
- Kersten, A., Poitschek, C. Rauch, S., and E. Aberer (1995). Effects of penicillin, ceftriaxone, and doxycycline on morphology of *Borrelia burgdorferi*. *Antimicrob. Agents. Chemother.* **39**, 1127-1133.
- Kollars, T.M., Kollars, P.G. Oliver, J.H. Masters E.J., and D. Miles (1997). Effects of two commonly used anesthetics on the *in vitro* growth of *Borrelia burgdorferi* and *B. garinii*. *J. Spirochet. Tick-borne. Dis.* **4**, 21-23.
- Kraiczy, P., Weigand, J. Wichelhaus, T.A. Heisig, P. Backes, H. Schäfer, V. Acker, G. Brade, V., and K.P. Hunfeld (2001). *In vitro* activities of fluoroquinolones against the spirochete *Borrelia burgdorferi*. *Antimicrob. Agents. Chemother.* **45**, 2486-2494.
- Kramer, M.D., Wallich, R., and M.M. Simon (1996). The outer surface protein A (OspA) of *Borrelia burgdorferi*: a vaccine candidate and bioactive mediator. *Infection.* **24**, 190-194.
- Lagal V., Postic D. Ružić-Sabljić E., and G. Baranton (2003). Genetic diversity among *Borrelia* strains determined by single-strand conformation polymorphism analysis of the ospC gene and its association with invasiveness. *J Clin Microbiol.* **41**, 5059-5065.
- Levin, J.M., Nelson, J.A. Segreti, J. Harrison, B. Benson, CA., and F. Strle (1993). *In vitro* susceptibility of *Borrelia burgdorferi* to 11 antimicrobial agents. *Antimicrob. Agents. Chemother.* **37**, 1444-1446.
- Lindgren, E., and T.G.T. Jaenson (2006). Lyme borreliosis in Europe: Influences of climate and climate change, epidemiology and adaptation measures, In: Climate change and adaptation strategies for human health (Eds. B. Menne and K.L. Ebi), 157-188. Steinkopff Verlag, Darmstadt.
- Luft, B.J., Gorevic, P.D. Halperin, J.J., Volkman DJ., and R.J. Dattwyler (1989). A perspective on the treatment of Lyme borreliosis. *Rev. Infect. Dis.* **11**, 1518-1525.

- Luft, B.J., Dattwyler, R.J. Johnson, R.C. Luger, S.W. Bosler, E.M. Rahn, D.W. Masters, E.J. Grunwaldt, E. and S.D. Gadgil (1996). Azithromycin compared with amoxicillin in the treatment of erythema migrans. A double-blind, randomized, controlled trial. *Ann. Intern. Med.* **124**, 785-791.
- Maraspin, V., Ružić-Sabljić, E. Strle, F. Cimperman, J. Jereb, M., and V. Preac-Mursic (1995). Persistence of *Borrelia burgdorferi* after treatment with antibiotics. *Alpe. Adria. Microbiol. J.* **3**, 211-216.
- Maraspin, V., Ružić-Sabljić, E. Cimperman, J. Lotrič-Furlan, S. Jurca, T. Picken R.N., and F. Strle (2001). Isolation of *Borrelia burgdorferi* sensu lato from blood of patients with erythema migrans. *Infection.* **29**, 65-70.
- Maraspin, V., Cimperman, J. Lotric-Furlan, S. Ruzić-Sabljić, E. Jurca, T., and F. Strle (2002). Cerebrospinal fluid findings in adult patients with multiple erythema migrans. *Wien. Klin. Wochenschr.* **114**, 505-509.
- Morgenstern, K., Baljer, G. Norris, D.E. Kraiczy, P. Hanssen-Hübert, C., and K.P. Hunfeld (2009). In vitro susceptibility of *Borrelia spielmanii* to antimicrobial agents commonly used for treatment of Lyme disease. *Antimicrob. Agents. Chemother.* **53**, 1281-1284.
- Nadelman, R.B., Nowakowski, J. Forseter, G. Goldberg, N.S. Bittker, S. Cooper, D. Aguero-Rosenfeld, M., and G.P. Wormser (1996). The clinical spectrum of early Lyme borreliosis in patients with culture-confirmed erythema migrans. *Am. J. Med.* **100**, 502-508.
- Nowakowski, J., Schwartz, I. Liveris, D. Wang, G. Aguero-Rosenfeld, M.E. Girao, G. McKenna, D. Nadelman, R.B. Cavaliere, L.F., and G.P. Wormser (2001). Laboratory diagnostic techniques for patients with early Lyme disease associated with erythema migrans: a comparison of different techniques. *Clin. Infect. Dis.* **33**, 2023-2027.
- Pfister, H.W., Preac-Mursic V. Wilske, B. Schielke, E. Sörgel, E., and K.M. Einhäupl (1991). Randomized comparison of ceftriaxone and cefotaxime in Lyme neuroborreliosis. *J. Infect. Dis.* **163**, 311-318.
- Picken, R.N., Cheng, Y. Strle, F. Cimperman, J. Maraspin, V. Lotrič-Furlan, S. Ružić-Sabljić E. Han, D. Nelson, J.A. Picken, M.M., and G.M. Trenholme (1996). Molecular characterization of *Borrelia burgdorferi* sensu lato from Slovenia revealing significant differences between tick and human isolates. *Eur. J. Clin. Microbiol. Infect. Dis.* **15**, 313-323.
- Pollack, R.J., Telford, S.R. 3rd, and A. Spielman (1993). Standardization of medium for culturing Lyme disease spirochetes. *J. Clin. Microbiol.* **31**, 1251-1255.
- Preac-Mursic, V., Wilske, B., and G. Schierz (1986). European *Borrelia burgdorferi* isolated from humans and ticks culture conditions and antibiotic susceptibility. *Zbl. Bakteriolog. Hyg. A.* **263**, 112-118.
- Preac-Mursic, V., Weber, K. Pfister, H.W. Wilske, B. Gross, B. Baumann, A., and J. Prokop (1989). Survival of *Borrelia burgdorferi* in antibioticly treated patients with Lyme borreliosis. *Infection.* **17**, 355-359.
- Preac-Mursic, V., Wilske, B., and S. Reinhardt (1991). Culture of *Borrelia burgdorferi* on six solid media. *Eur. J. Clin. Microbiol. Infect. Dis.* **10**, 1076-1079.
- Preac-Mursic, V., and B. Wilske (1993). Biology of *Borrelia burgdorferi*, In: Aspects of Lyme borreliosis (Eds. K. Weber and W. Burgdorfer), 44-58. Springer Verlag, Berlin.
- Preac-Mursic, V., Marget, W. Busch, U. Pleterski-Riegler, D., and S. Hagel (1996a). Kill kinetics of *Borrelia burgdorferi* and bacterial findings in relation to the treatment of Lyme borreliosis. *Infection.* **24**, 9-16.
- Preac-Mursic, V., Wanner, G. Reinhardt, S. Wilske, B. Busch, U., and W. Margret (1996b). Formation and cultivation of *Borrelia burgdorferi* spheroplast-L form variants. *Infection.* **24**, 218-226.
- Reisinger E., Wendelin, I., and R.Gasser (1995). Inactivation of diaminopyrimidines and sulfonamides in Barbour-Stoenner-Kelly medium for isolation of *Borrelia burgdorferi*. *Eur. J. Clin. Microbiol. Infect. Dis.* **14**, 732-733.
- Reisinger, E., Wendelin, I. Gasser, R. Halwachs, G. Wilders-Truschnig, M., and G. Krejs (1996). Antibiotics and increased temperature against *Borrelia burgdorferi* in vitro. *Scand. J. Infect. Dis.* **28**, 155-157.
- Rijpkema, S.G., Tazelaar, D.J., Molkenboer, M.J., Noordhoek, G.T., Plantinga, G., Schouls, L.M., and J.F. Schellekens (1997). Detection of *Borrelia afzelii*, *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* and group VS116 by PCR in skin biopsies of patients with erythema migrans and acrodermatitis chronica atrophicans. *Clin. Microbiol. Infect.* **3**, 109-116.
- Rosa, P.A., Tilly, K., and P.E. Stewart (2005). The burgeoning molecular genetics of the Lyme disease spirochaete. *Nat. Rev. Microbiol.* **3**, 129-43.
- Ružić-Sabljić, E., Maraspin, V. Lotrič-Furlan, S. Jurca, T. Logar, M. Pikelj-Pečnik, A., and F. Strle (2002). Characterization of *Borrelia burgdorferi* sensu lato strains isolated from human material in Slovenia. *Wien. Klin. Wochenschr.* **114**, 544-550.
- Ružić-Sabljić, E., and F. Strle (2004). Comparison of growth of *Borrelia afzelii*, *B. garinii*, and *B. burgdorferi* sensu stricto in MKP and BSK-II medium. *Int. J. Med. Microbiol.* **294**, 407-412.
- Ružić-Sabljić, E., Podreka, T. Maraspin V., and F. Strle (2005). Susceptibility of *Borrelia afzelii* strains to antimicrobial agents. *Int. J. Antimicrob. Agents.* **25**, 474-478.

- Ružić-Sabljić, E., Lotrič-Furlan, S., Maraspin, V., Cimperman, J., Logar, M., Jurca, T., and F. Strle (2006). Comparison of isolation rate of *Borrelia burgdorferi* sensu lato in MKP and BSK-II medium. *Int. J. Med. Microbiol.* **296**, 267-273.
- Saint Girons, I., Norris, S.J., Göbel, U., Meyer, J., Walker, E.M., and R. Zuerner (1992). Genome structure of spirochetes. *Res. Microbiol.* **143**, 615-621.
- Schwan, T.G., Piesman, J., Golde, W.T., Dolan, M.C., and P.A. Rosa (1995). Induction of an outer surface protein on *Borrelia burgdorferi* during tick feeding. *Proc. Natl. Acad. Sci. US A.* **92**, 2909-2913.
- Schwan, T.G. (2003). Temporal regulation of outer surface proteins of the Lyme-disease spirochaete *Borrelia burgdorferi*. *Biochem. Soc. Trans.* **31**, 108-112.
- Seinost, G., Dykhuizen, D.E., Dattwyler, R.J., Golde, W.T., Dunn, J.J., Wang, I.N., Wormser, G.P., Schriefer, M.E., and B.J. Luft (1999). Four clones of *Borrelia burgdorferi* sensu stricto cause invasive infection in humans. *Infect. Immun.* **67**, 3518-3524.
- Sickliger, M., Wienecke, R., and U. Neubert (2003). *In vitro* susceptibility testing of four antibiotics against *Borrelia burgdorferi*: a comparison of results for the three genospecies *Borrelia afzelii*, *Borrelia garinii*, and *Borrelia burgdorferi* sensu stricto. *J. Clin. Microbiol.* **41**, 1791-1793.
- Smith, R.P., Schoen, R.T., Rahn, D.W., Sikand, V.K., Nowakowski, J., Parenti, D.L., Holman, M.S., Persing, D.H. and A.C. Steere (2002). Clinical characteristics and treatment outcome of early Lyme disease in patients with microbiologically confirmed erythema migrans. *Ann. Intern. Med.* **136**, 421-428.
- Stanek, G., and F. Strle (2003). Lyme borreliosis. *Lancet.* **362**, 1639-1647.
- Steere, A.C. (2001). Lyme disease. *N. Engl. J. Med.* **345**, 115-125.
- Steere, A.C., Coburn, J., and L. Glickstein (2004). The emergence of Lyme disease. *J. Clin. Invest.* **113**, 1093-1101.
- Stiernstedt, S.H., Tadesse, T., and B. Wretling (1999). Dialysis culture for determination of MIC and MBC of benzylpenicillin against *Borrelia burgdorferi*. *APMIS.* **107**, 380-382.
- Straubinger, R.K., Summers, B.A., Chang, Y.F., and M.J. Appel (1997). Persistence of *Borrelia burgdorferi* in experimentally infected dogs after antibiotic treatment. *J. Clin. Microbiol.* **35**, 111-116.
- Strle F, Preac-Mursic V, Cimperman J, Ruzic E, Maraspin V, and M. Jereb (1993). Azithromycin versus doxycycline for treatment of erythema migrans: clinical and microbiological findings. *Infection.* **21**, 83-88.
- Strle, F., Nelson, J.A., Ružić-Sabljić, E., Cimperman, J., Maraspin, V., Lotrič-Furlan, S., Cheng, Y., Picken, M.M., Trenholme, G.M., and R.N. Picken (1996a). European Lyme borreliosis: 231 culture-confirmed cases involving patients with erythema migrans. *Clin. Infect. Dis.* **23**, 61-65.
- Strle, F., Maraspin, V., Lotric-Furlan, S., Ružić-Sabljić, E., and J. Cimperman (1996b). Azithromycin and doxycycline for treatment of *Borrelia* culture-positive erythema migrans. *Infection.* **24**, 64-68.
- Strle, F., Nadelman, R.B., Cimperman, J., Nowakowski, J., Picken, R.N., Schwartz, I., Maraspin, V., Aguero-Rosenfeld, M.E., Varde, S., Lotrič-Furlan S., and G.P. Wormser (1999a). Comparison of culture-confirmed erythema migrans caused by *Borrelia burgdorferi* sensu stricto in New York State and by *Borrelia afzelii* in Slovenia. *Ann. Intern. Med.* **130**, 32-36.
- Strle, F. (1999b). Principles of the diagnosis and antibiotic treatment of Lyme borreliosis. *Wien. Klin. Wochenschr.* **111**, 911-915.
- Strle, F., Ružić-Sabljić, E., Logar, M., Maraspin, V., Lotrič-Furlan, S., Cimperman, J., Ogrinc, K., Stupica, D., Nadelman, R.B., Nowakowski, J., and G.P. Wormser (2011). Comparison of erythema migrans caused by *Borrelia burgdorferi* and *Borrelia garinii*. *Vector. Borne. Zoonotic. Dis.* **11**, 1253-1258.
- Strle, K., Drouin, E.E., Shen, S., Houry, J.E., McHugh, G., Ružić-Sabljić, E., Strle, F., and A.C. Steere (2009). *Borrelia burgdorferi* stimulates macrophages to secrete higher levels of cytokines and chemokines than *Borrelia afzelii* or *Borrelia garinii*. *J. Infect. Dis.* **200**, 1936-1943.
- Terekhova, D., Sartakova, M.L., Wormser, G.P., Schwartz, I., and F.C. Cabello (2002). Erythromycin resistance in *Borrelia burgdorferi*. *Antimicrob. Agents. Chemother.* **46**, 3637-3640.
- Thanassi, W.T., and R.T. Schoen (2000). The Lyme disease vaccine: conception, development, and implementation. *Ann. Intern. Med.* **132**, 661-668.
- Wang, G., van Dam, A.P., Schwartz, I., and J. Dankert (1999). Molecular typing of *Borrelia burgdorferi* sensu lato: taxonomic, epidemiological, and clinical implications. *Clin. Microbiol. Rev.* **12**, 633-653.
- Wilske, B., Preac-Mursic, V., Schierz, G., Kühbeck, R., Barbour, A.G., and M. Kramer (1988). Antigenic variability of *Borrelia burgdorferi*. *Ann. N.Y. Acad. Sci.* **539**, 126-143.
- Wilske, B., and V. Preac-Mursic (1993). Microbiological diagnosis of Lyme borreliosis, In: Aspects of Lyme borreliosis (Eds. K. Weber, W. Burgdorfer, and G. Shiery), 268-299. Springer-Verlag, Berlin.
- Wilske, B., and H.W. Pfister (1995). Lyme borreliosis research. *Cur. Opin. Inf. Dis.* **8**, 137-144.
- Wilske, B., Busch, U., Fingerle, V., Jauris-Heipke, S., Preac-Mursic, V., Rössler, D., and G. Will (1996). Immunological and

- molecular variability of OspA and OspC. Implications for *Borrelia* vaccine development. *Infection*. **24**, 208-212.
- Wormser, G.P., Bittker, S. Cooper, D. Nowakowski, J. Nadelman, R.B., and C. Pavia (2000a). Comparison of the yields of blood cultures using serum or plasma from patients with early Lyme disease. *J. Clin. Microbiol.* **38**, 1648-1650.
- Wormser, G.P., Nadelman, R.B. Dattwyler, R.J. Dennis, D.Z. Shapiro, E.D. Stere, A.C. Rish, T.J. Rahn, D.W. Coyle, P.K. Persing, D.H. Fish, D., and B.J. Luft (2000b). Practice guidelines for the treatment of Lyme disease. *Clin. Infect. Dis.* **31**, 1-14.
- Wormser, G.P., Ramanathan, R. Nowakowski, J. McKenna, D. Holmgren, D. Visintainer, P. Dornbush, R. Singh, B., and R.B. Nadelman (2003). Duration of antibiotic therapy for early Lyme disease. A randomized, double-blind, placebo-controlled trial. *Ann. Intern. Med.* **138**, 697-704.
- Xu, Y., and R. C. Johnson (1995). Analysis and comparison of plasmid profiles of *Borrelia burgdorferi* sensu lato strains. *J. Clin. Microbiol.* **33**, 2679-2685.
- Yang, X., Popova, T. G. Goldberg, M. S., and M. V. Norgard (2001). Influence of cultivation media on genetic regulatory patterns in *Borrelia burgdorferi*. *Infect. Immun.* **69**, 4159-4163.

